# Traumatic Brain Injury Causes the Formation of Rod Microglia/Macrophages in the Cortex

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## Introduction

Traumatic brain injury (TBI) results when abnormal force to the skull causes either gross or microscopic disruption of brain structure. This injury can result in edema, inflammation, and acute behavioral disturbance. The vast majority of TBIs are mild to moderate in nature, from which patients recover quickly. There is evidence, however, that glial activation and inflammatory processes persist long after the initial recovery phase (1). Microglia, the innate immune cells resident to the brain, mediate both acute inflammation and chronic inflammation. Chronic inflammation, marked by prolonged heightened cytokine levels and altered glial morphology, is less understood but is thought to underlie the increased risk of depression and neurodegenerative complications that patients face long after TBI (2, 3). After diffuse injury in a murine model, microglia are in a primed state, where peripheral immune challenge causes exaggerated cytokine release and depressive-like behaviors (4). Microglia maintained activated morphologies up to 12 months after focal injury, which corresponds with cognitive deficits (5). Together these findings support the idea that chronic microglial dysfunction after TBI can precipitate behavioral dysfunction long after initial recovery from injury. Therefore, understanding the functional role of microglia in the sub-acute window, when acute inflammation is waning and chronic inflammation is developing, will provide critical insight into the mechanisms behind behavioral dysfunction long after injury.

Microglia have been shown to take on an elongated bipolar or rod-shaped morphology and form trains in the cerebral cortex after diffuse TBI in rodents (6, 7). The number of rod microglia peaks 7d after injury and persists up to 28d in rats and these cells upregulate markers of inflammatory activation including MHCII and CD68 (6, 7). At homeostasis, microglia have very small cell bodies and extend long, thin processes in all directions in order to survey the surrounding microenvironment (8). Thus, the rod morphology is distinctive and unique to pathologic states. In fact, isolated rod microglia are observed in humans following chronic viral infection, in pigs following prenatal infection (9), and in mice after toxin-induced hippocampal lesion (10). Optic nerve transection causes the formation of rod microglia in the retina and, similar to TBI, these cells form long trains (11). In this context, the rod microglia are proliferating after injury to form trains (11). An *in vitro* assessment of rod microglia also found them to proliferate and to have an anti-inflammatory, reparative gene expression profile (12). Together, these studies suggest that pathological conditions cause formation of activated rod microglia; however, the functional significance of these cells *in vivo* after TBI is unknown.

In the present study, we aimed to further characterize the alignment, origin, and inflammatory capacity of rod microglia in the context of diffuse TBI. We first show novel data that rod microglia can align with vascular structures. We also show that rod microglia are resident microglia, not peripheral macrophages that have infiltrated the brain parenchyma. Lastly, we show that anti-inflammatory intervention with methylene blue improves functional recovery and reduces the overall number of rod microglia, suggesting a potential pro-inflammatory role for these cells in the context of TBI.

**Methods**

## Animals

Adult (3 mo.) male mice were obtained from Charles River Laboratories and individually housed in a vivarium at Ohio State University. Mice were kept on a 12h light/dark cycle with ad libitum access to food and water. Mice for chimera experiment were C57BL/6, while all other experiments used BALB/c mice.

## Midline fluid percussion injury

Mice were given a moderate and diffuse TBI using a midline fluid percussion injury apparatus (Custom Design and Fabrication, Richmond, VA). Our lab has published using this model preiously (4, 13, 14). In brief, mice were deeply anesthetized with isoflurane, a small craniectomy was made at midline, and a Luer-lock hub was affixed to the skull. The dura is not disrupted during this procedure and mice were returned to home cages to recover. After mice recovered from the initial surgery (2-3h), they are briefly anesthetized with isoflurane, attached to the fluid percussion apparatus, and a fluid pulse was applied to the surface of the dura. Following injury, the scalp was closed and the time before the mouse self-righted was measured. Righting reflex times between 3-9min are considered moderate injuries, and only mice with this level of injury were included in the study. Sham controls underwent the same surgical procedures without the fluid pulse.

## GFP bone marrow chimeras

GFP chimeras were generated as previously described (15). In brief, recipient C57BL/6 adult male mice were injected intraperitoneally on two consecutive days wih busulfan (30 mg/kg in 1:1 DMSO and $H_2O$) to ablate the bone marrow (BM). The following day, BM was harvested from the femur of a C57BL/6-$Tg^{(CAG\text{-}EGFP)}$ mouse, in which GFP is expressed in all cell types

(Jackson Laboratories, catalog # 006567). BM-derived cells ($1\times10^6$) were injected intravenously (iv) into the recipient mice and mice were given 4 weeks for engraftment and recovery. Extent of chimerism was confirmed by flow cytometry.

Methylene blue administration

Methylene blue (Sigma Aldrich) was administered 15min, 12h, and 24h after TBI at a dose of 2mg/kg iv as previously published by our lab (13).

Rotarod

Motor coordination was assessed by performance on rotarod (Rotamex, Columbus, OH) (4). Mice were placed on the run for three trials per day, for three consecutive days prior to injury (training) and each day after TBI or sham-injury (testing). The rod accelerated at a constant speed and latency to fall was recorded.

Tissue collection and immunohistochemistry

At 7 days post-injury, mice were perfused with PBS followed by 4% paraformaldehyde (PFA) solution (4). Brains were removed and post-fixed in 4% PFA overnight and then placed in 30% sucrose solution for 48h. Tissue was flash-frozen in -80°C isopentane, cryosectioned at 30μm thickness, and stored in cryoprotectant at -20°C until labeling. Sections were blocked with 5% normal donkey serum (NDS) in 1% bovine serum albumin (BSA) in PBS prior to incubation with primary antibodies diluted 1:1000 in blocking solution overnight at 4°C (Iba1, CD45, Ly6C). Appropriate secondaries were diluted 1:500 in 1% BSA in PBS and incubated for 2h at room temperature.

**Results**

Our findings confirm that midline fluid percussion injury causes the formation of rod microglia in the cortex 7dpi. These cells form perpendicular to the cortical surface and can form long trains (Figure 1A). Furthermore, trains often occur near areas of focal microglial activation in cortical layer V (Figure 1B-C). Double labeling with Iba1 (microglia) and Ly6C (blood vessels) shows that rod microglia form along large blood vessels when present in the cortex (Figure 2A-C). Rod microglia do not seem to align with smaller capillaries, which are tortuous in shape rather than straight.

In order to determine if these vascular-aligned cells might be peripheral macrophages, we double labeled for Iba1 and CD45, a marker of monocytes and macrophages. Both activated microglia in cortical layer V and rod microglia are Iba1+/CD45+ (Figure 3A). These are in contrast to Iba1-/CD45+ monocytes (Figure 3B). Because macrophages upregulate Iba1 and downregulate CD45 when entering the brain parenchyma, GFP bone marrow chimeras were generated in order to differentiate peripheral cells from resident microglia. In the majority of chimeras, rod microglia were Iba1+/GFP- (Figure 4A). In mice that had significant GFP+ cell trafficking into the parenchyma, those cells formed both rod shapes and trains (Figure 4B).

Our last experiment was aimed at determining if anti-inflammatory intervention with methylene blue (MB) prevented the formation of rod microglia. MB intervention improves recovery of motor coordination after injury (Figure 5A). This was associated with a reduction in the number of rod microglia counted on representative images (Figure 5B-E).

**Discussion**

The finding that rod microglia can align with large blood vessels after TBI is novel, as these cells were previously thought only to align with neuronal structures. Therefore it is likely that these

cells are capable of aligning with both of these structures. It is possible that differing cues from within the injured cortex. For instance, alignment with blood vessels may be a result of endothelial or circulating cytokine signals, whereas neuronal injury may signal microglia to align with damaged axons. These differing alignments may also hint to differing functional roles. For instance, perhaps vascular-aligned rods maintain blood-brain-barrier integrity whereas neuronal-aligned rods have a reparative role. Understanding the differing roles of rod microglia in the cortex is important to determining potential therapeutic targets. Because MB intervention prevented formation of some, but not all rod microglia, it is possible that anti-inflammatory intervention is more effective at preventing the formation of rod microglia along vasculature but not neuronal structures. Further characterization is necessary to understand how targeting these cells may have therapeutic benefit after TBI.

The confirmation that these cells are in fact resident microglia is significant. CD45 upregulation on microglia does not occur in other models that induce microglial activation, such as stress(16). This may suggest that injury not only changes the shape of microglia, but significantly alters the inflammatory profile of resident glia. Furthermore, TBI literature often fails to rigorously differentiate peripheral macrophages from resident microglia. Here, with the use of GFP chimeras, we definitively show that these are resident microglia. In other conditions where rod microglia form, it has been shown that rod cells are the result of microglial proliferation (11, 12). This is an important future direction for our work.

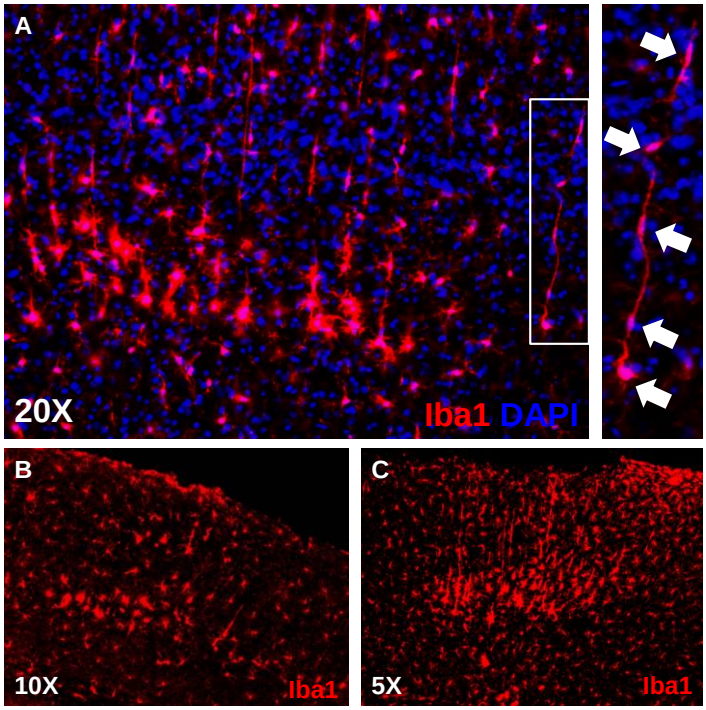


**Figure 1: Rod microglia form trains perpendicular to the cortical surface near focal activation in cortical layer V.** Adult male BALB/c mice were given sham-surgery or moderate TBI. Mice were perfused with 4% paraformaldehyde (PFA) 7 days post-injury and tissue was labeled for Iba1 and stained with DAPI to show nuclei. Rod microglia form trains in the cortex (A) and often appear near areas of focal microglia activation in cortical layer V (A-C).

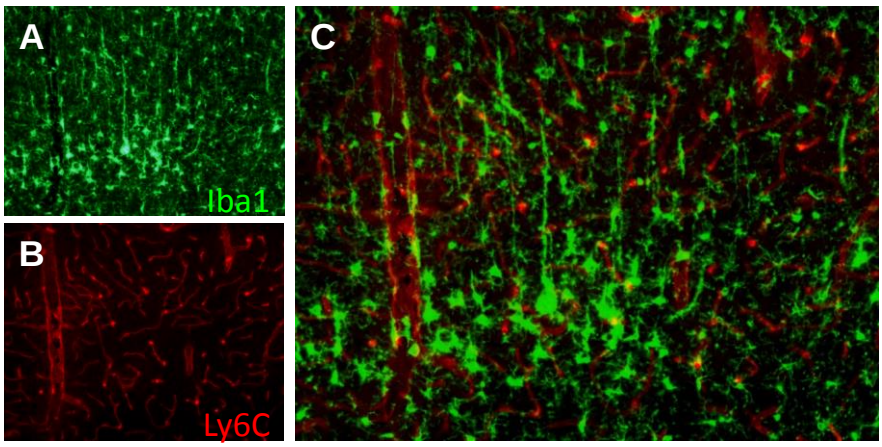


**Figure 2: Rod-shaped and activated microglia align with large blood vessels in the cortex .** Adult male BALB/c mice were given sham-surgery or moderate TBI. Mice were perfused with 4% paraformaldehyde (PFA) 7 days post-injury and tissue was labeled for Iba1 (A) and Ly6C (B). Overlay of these images (C) show that some, but not all, trains of microglia align with medium-sized blood vessels within the cortex.

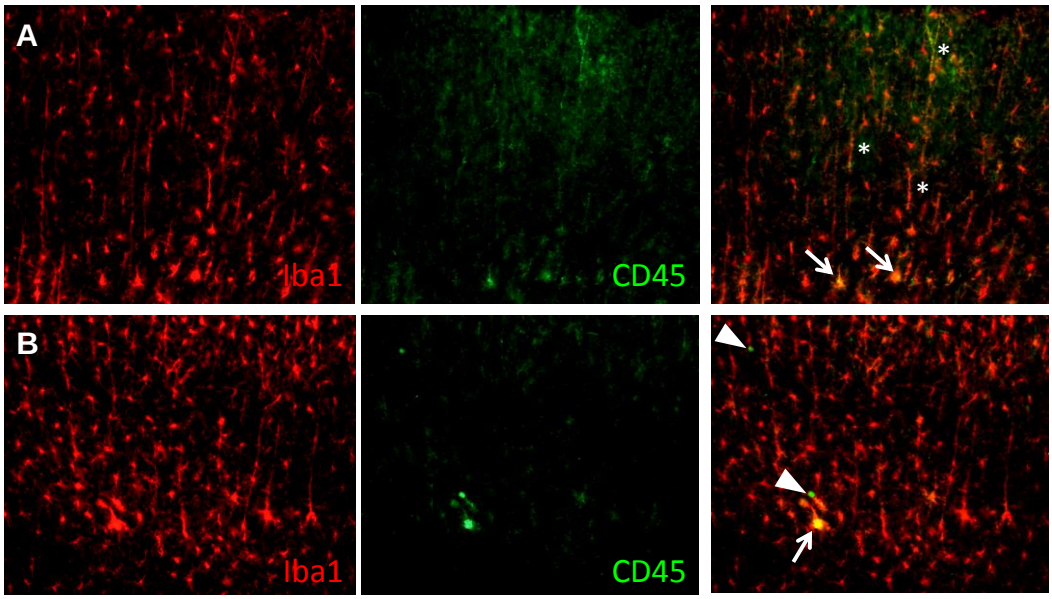


**Figure 3: Rod-shaped microglia upregulate CD45.** Adult male BALB/c mice were given sham-surgery or moderate TBI. Mice were perfused with 4% paraformaldehyde (PFA) 7 days post-injury and tissue was labeled for Iba1 and CD45. Overlay of these images show that both rod microglia (asterisk) and locally activated/deramified microglia (arrow) are Iba1+/CD45+. Peripheral monocytes (arrowhead) are Iba1-/CD45+. (A) and (B) are representative images from two different animals.

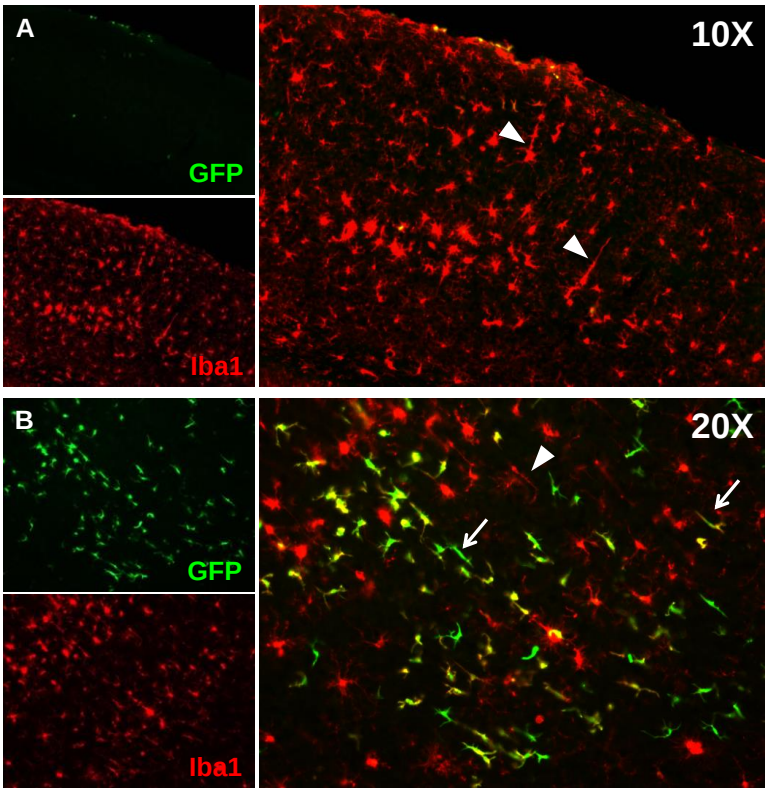

**Figure 4: Macrophages can take on rod-shaped morphologies in the cortex after TBI.** Adult male C57BL/6 GFP bone marrow chimeras were given TBI and 7d later were perfuse-fixed with 4% PFA, sectioned, and labeled for Iba1. Both Iba1+/GFP- (arrowhead) and Iba1+/GFP+ (arrow) rod-shaped myeloid cells are seen in the cortex after TBI. (A) and (B) are representative images from two different animals.

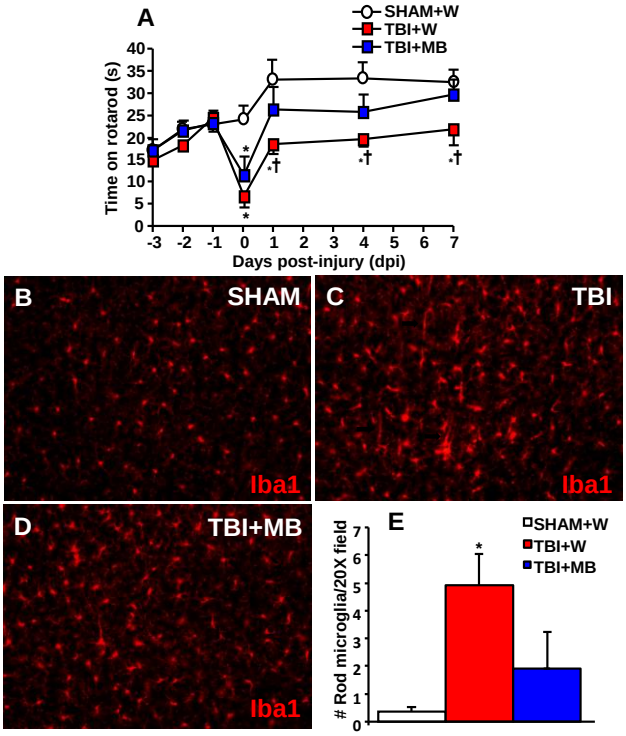


**Figure 5: Acute methylene blue intervention after TBI reduces rod microglia and improves functional recovery.** Adult male BALB/c were subjected to sham-injury or TBI. Methylene blue (MB) was injected intravenously 15m, 12h, and 24h after injury. Mice were trained on the rotarod 3d prior to injury and tested daily until 7dpi when mice were perfuse-fixed with 4% PFA, sectioned, and labeled for Iba1. Images of the cortex at midline were taken at 20X magnification (B-D). Methylene blue administration improved performance on the rotarod (A) and reduced the number of rod microglia (E). * denotes $p < 0.05$ from SHAM+W and † denotes $p < 0.10$ from TBI+MB.

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